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13. ABSTRACT (Maximum 200 Words) Monitoring fluorescently tagged proteins in live cells enables the observation of spatial and temporal events in a way that would otherwise not be possible. We are using this approach to examine normal and tumor human mammary epithelial cells (growing in 2D and 3D cultures) before and after addition of exogenous agents. We used confocal microscopy to examine living cells containing proteins tagged with green fluorescent protein (GFP). We examined GFP-tagged cytoskeletal proteins, those proteins responsible for cell shape and transport within the cell, and GFP-tagged proteins of the Wnt signaling pathway, a pathway involved in cancer. Normal cells demonstrate slow, directional movements across the dish whereas tumor cells demonstrate rapid and exaggerated, random movements. Cells containing actin-GFP show that tumor cells, unlike normal cells, have excessive actin-mediated ruffles, filopodia, and microspikes. Cells containing GFP-tagged β -catenin, a Wnt signaling protein, become rounded and demonstrate large aggregates of β -catenin in the cell nucleus, consistent with reports that β -catenin can activate gene transcription. The cells with β -catenin-GFP also demonstrate numerous protrusions ("blebs") emanating from the cell surface. These protrusions are rapidly extending and contracting as the cells make random movements around the dish.				
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FOREWORD

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X^{cal} For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

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N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

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Charles Karabell 7/30/00
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(5) INTRODUCTION

The goal of this research is to develop a technique for imaging living human breast epithelial cells in 3-D cultures and to evaluate their responses to the application of exogenous factors. Most model systems used to study breast cancer utilize cells growing in monolayers on plastic substrates. Although a great deal of information about cells and their responses to exogenous agents, such as therapeutic drugs, can be learned from these studies, there are also major limitations to this approach. In short, cells growing on plastic are flat, whereas cells in the body are very three-dimensional. Recent data from a number of laboratories demonstrate that cells growing in monolayers do not necessarily respond to exogenous substances in the same manner as do cells growing in 3-D (Weaver et al., 1997). Therefore, we are developing the technology for imaging human mammary epithelial cells growing in a three-dimensional reconstituted basement membrane. This technique will enable the monitoring of fluorescently labeled proteins in living cells and will provide a way to evaluate "normal," premalignant, and tumor cells. Using this approach we will be able to detect rapid, "real-time" responses by these cells to the effects of a spectrum of exogenous factors, including therapeutic agents.

(6) BODY

Specific Aim 1: Visualization Of Living Human Mammary Epithelial Cells Growing In A Three-Dimensional Matrix.

Human mammary epithelial cells growing in three-dimensional reconstituted basement membrane components (i.e. Matrigel) are very difficult to examine in the living state using confocal microscopy due to the fact that they are very thick ($> 200 \mu\text{m}$). Our initial attempts to examine these cultures, which were done by imaging living cells that had been labeled with DiOC₆(3), determined that the thickest cultures we could successfully image with the confocal microscope were $\leq 50 \mu\text{m}$ thick. Therefore, we modified our 3-D cultures accordingly using a method in which the Matrigel was dripped onto cells growing in 2-D, allowing them to grow into the Matrigel and form thinner 3-D cultures. Unfortunately, our attempts to examine individual protein constructs tagged with green fluorescent protein (GFP) in these cultures presented numerous difficulties and were also unsuccessful. The protein constructs tagged with GFP that we examined were much more difficult to detect than was the lipophilic dye, DiOC₆(3), which labels numerous organelle membranes and glows very brightly. The β -catenin-GFP protein constructs we wanted to examine could not be visualized at low laser intensity, and the use of more intense laser beams required to see them in 3-D were damaging to the cells. My laboratory has recently purchased a multi-photon (2-photon) microscope, which has the capacity to examine 3-D cultures of human mammary epithelial cells growing in Matrigel $500 \mu\text{m}$ thick (we tested this on the Zeiss 510 NLO system that we ultimately purchased). This imaging system will be delivered in September 2000, so these studies have been postponed until its arrival. In the interim, we are examining GFP protein constructs in the same cells growing in 2-D cultures (on plastic) to address those questions regarding cellular differences in 2-D vs. 3-D cultures and for testing of the effects of exogenous factors on cell growth.

We are examining the behavior of proteins of the Wnt signaling pathway, which is an important pathway for normal embryonic development and has also been implicated in cancer. Normal development relies on translocation of one protein in this pathway, β -catenin, to the nucleus where it turns on gene transcription (Larabell et al., 1997; Molenaar et al., 1996). In cancer, mutations in three of the regulatory genes in the Wnt pathway have been identified in primary cancers and several others can induce cancers in rodents. All of these mutations are associated with accumulation of β -catenin in the nucleus of the tumor cells and activation of gene transcription (Polakis, 2000). Therefore, we are monitoring the behavior of β -catenin-GFP in normal and tumor human mammary epithelial cells growing in 2-D and 3-D. The ability to monitor movement of β -catenin-GFP in to or out of the nucleus upon the addition of an exogenous agent would aid in testing the efficacy of that agent as a potential chemotherapeutic agent.

For these studies, GFP constructs of the protein β -catenin were introduced into the cells using electroporation (Bio-Rad GenePulser II) with a voltage of 0.25 kV and 5 μ F for approximately 23 seconds (pulse is determined by GenePulser). In each case, 100 μ g plasmid DNA were added to 3×10^6 cells in 400 μ l total volume of DMEM/F12. After electroporation cells were plated at 1×10^6 cells per 35 mm plate in environmental viewing dishes obtained from Bioprotech (Butler, PA). The cells were then examined with the confocal microscope, where multiple images of a single optical section were collected at intervals ranging from 10 sec – 30 min. To reduce the possibility of laser damage, laser intensity was kept at less than 10% of maximum. Data were analyzed using ImageSpace software (Molecular Dynamics) on a Silicon Graphics computer.

In normal human mammary epithelial cells (HMT225, S-1), β -catenin-GFP becomes localized in the cytoplasm, but not the nucleus (Fig. 1), which is consistent with results obtained from immunocytochemical analyses. In tumor cells (HMT225, T-4), however, β -catenin-GFP becomes localized in the nucleus of cells growing in 2-D, which is not consistent with results obtained from immunocytochemical analyses. Immunolocalization analyses of cells growing in 3-D (in Matrigel), however, do reveal the presence of β -catenin in the nucleus (Larabell, Weaver and Bissell, unpublished data). The reason for the difference between the immunolocalization results and GFP results is being investigated. It may reflect the difference between endogenous and exogenous protein and demonstrate that excess β -catenin in the cell translocates to the nucleus. However, it might also reveal an inability of the antibodies to enter the nucleus of whole cells and recognize the antigen in the nucleus. Studies to differentiate between these two possibilities are in progress.

In those cells imaged early after transfection with β -catenin-GFP, small foci of β -catenin are seen in the nucleus (Fig. 2). At later time points, however, high levels of β -catenin-GFP are seen in the cell cytoplasm and large clusters of β -catenin-GFP are seen in the nucleus of these cells. The cells containing large amounts of β -catenin-GFP become rounded and demonstrate several projections from the cell surface that extend and retract at multiple sites around the cell. These cells are also quite motile, moving in a random fashion without directionality (Fig. 3).

Specific Aim #2: Examination of the effects of exogenous factors on living human mammary epithelial cells growing in a three-dimensional matrix.

Metastasis is a major problem associated with cancer and is related to the ability of cells to migrate. We are studying this process by examining cells that have been transfected with GFP-actin (normal and tumor cells) in order to study the effects of exogenous agents on migration and metastasis. Tumor cells demonstrate more random and dynamic behaviors than do normal cells. Normal cells crawl very slowly, typically in a single direction. Tumor cells, on the other hand, crawl ten times faster and frequently move in several different directions throughout the course of an experiment. The tumor cells often have lamellipodia located at the leading edge, or at both ends, or surrounding the entire cell; these lamellipodia demonstrate extensive ruffling activity. (Fig. 4). We are examining the effects of a unique plant compound on the motility of these cells by monitoring the behavior of living cells, normal and tumor, before and after addition of these compounds. Preliminary data indicates that this compound halts the migratory behavior of the tumor cells. (A patent application is being prepared.)

(7) KEY RESEARCH ACCOMPLISHMENTS:

- We have demonstrated the ability to image live cells that have been transfected with GFP protein constructs.
- We show that β -catenin-GFP translocates to the nucleus in 2-D cultures of live human mammary epithelial tumor cells, but not normal cells.
- We show that by monitoring cells transfected with actin-GFP, we can examine the effect of exogenous agents on cell migration of tumor vs. normal cells.

(8) REPORTABLE OUTCOMES

There are no publishable outcomes at this time. We have had difficulties doing the experiments with our confocal microscope. We have ordered a new, multi-photon (2-photon) microscope that will enable us to do the proposed experiments. In the interim, we are doing preliminary studies of live cells growing in 2-D. These studies have led to very preliminary data indicating that addition of an exogenous agent (a plant compound) halts migratory behavior of cells. Further studies are in progress and a patent application is being prepared.

(9) CONCLUSIONS

Live cell imaging of cells reveals data that could not readily be obtained using other techniques. We are developing such techniques for imaging human mammary epithelial cells growing in 3-D cultures. We expect this approach will yield valuable information about the responses of tumor cells to exogenous agents that would not otherwise be generated from studying fixed specimens. Studying cells growing in 3-D cultures in the living state is not trivial and has required numerous modifications of the cell growth conditions. (We are also purchasing a new multi-photon microscope that will facilitate

examination of very thick (500 μm) cell cultures.) Obtaining information about the efficacy of cell changes has been complicated by the fact that these cells grow very slowly and considerable time passes between making an adjustment and evaluating the consequences. Nonetheless, we have made considerable progress in this regard and have obtained preliminary data monitoring β -catenin-GFP in living breast cells, both normal and tumor. Once we understand the behavior of this protein in the cells growing in 2-D and 3-D cultures, we can begin to evaluate its responses to the addition of components expected to modify its distribution in cells. These studies will lay the foundation for using this system to investigate potential therapeutic agents.

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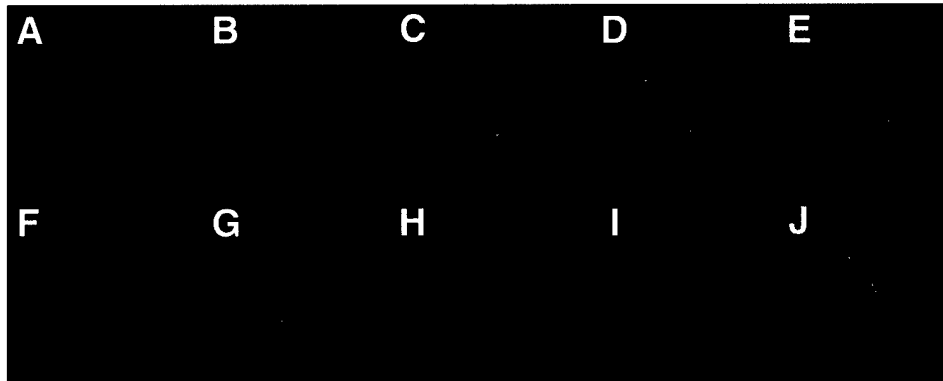


Figure 1. Normal human mammary epithelial cell transfected with GFP-tagged β -catenin shows accumulation of β -catenin in the cell cytoplasm but not the nucleus. Images were collected at 10-sec intervals, from A-J.

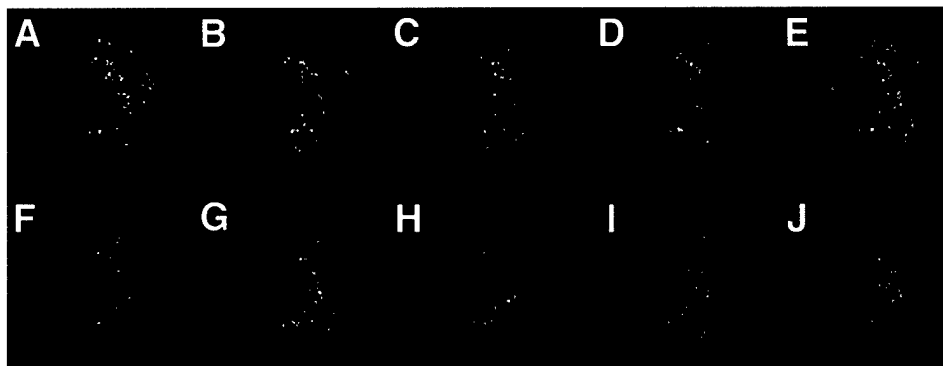


Figure 2. Human mammary epithelial tumor cell transfected with GFP-tagged β -catenin. Small foci of β -catenin-GFP are seen in the nucleus within 3-4 hours after electroporation. Images were collected at 10-sec intervals, from A-J.

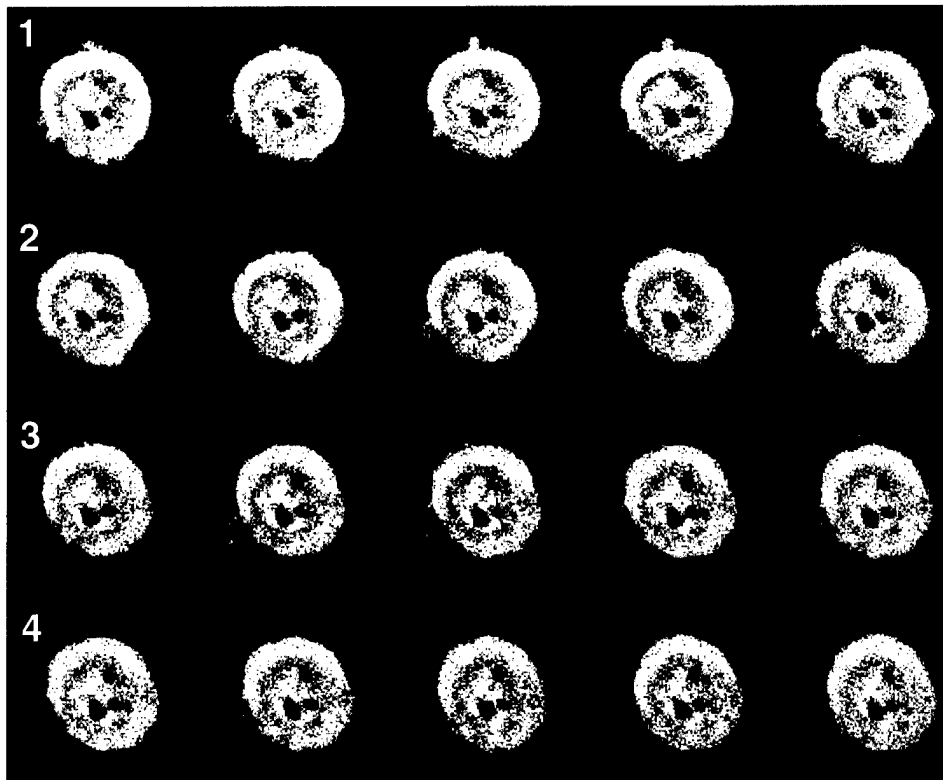


Figure 3. Human mammary epithelial tumor cell transfected with GFP-tagged β -catenin. At 8 hours after electroporation, β -catenin is seen in the cytoplasm; in addition, large clusters of β -catenin are seen in the nucleus. Membrane projections extend and retract at random sites around the cell. Images were collected at 10-sec intervals across rows, starting at row 1 and ending with row 4.



Figure 4. Human mammary epithelial tumor cell transfected with actin-GFP demonstrates filopodial extensions from random sites around the cell. Images were collected at 10-sec intervals from A-J.

(11) APPENDICES

NA

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